(12) UK Patent Application (19) GB (11) 2 216 416 (13) A

(43) Date of A publication 11.10.1989

(21) Application No 8905179.1

(22) Date of filing 07.03.1989

(30) Priority data
(31) [illegible] (32) 11.03.1988 (33) US

(71) Applicant
Sandoz Ltd

(Incorporated in Switzerland)

35 Lichtstrasse, CH-4002 Basle, Switzerland

(72) Inventors
Frederick B. Rudolph
Charles T. Van Buren

(74) Agent and/or Address for Service
B A Yorke & Co
Coomb House, 7 St. John's Road, Isleworth, Middlesex, TW7 6NH, United Kingdom

(51) INT CL[4]
A61K 31/675 31/505

(52) UK CL (Edition J)
A5B BHA B23Y B230 B26Y B261 B30Y B303
U1S S2411

(56) Documents cited
GB 2152814 A GB 2140880 A GB 1551179 A
GB 1540322 A GB 1353398 A GB 1270049 A
GB 1254744 A GB 1229008 A GB 1187830 A
GB 0971909 A EP 0196530 A EP 0112632 A
EP 0031285 A

(58) Field of search
UK CL (Edition J) A5B BHA
DIALOG computer search in WPI database

(54) Immunostimulating compositions comprising nucleobase sources

(57) The invention provides a method of stimulating the immune function with the aid of a nucleobase source, the use of nucleobase sources for immunostimulation and compositions comprising such nucleobase sources.

GB 2 216 416 A

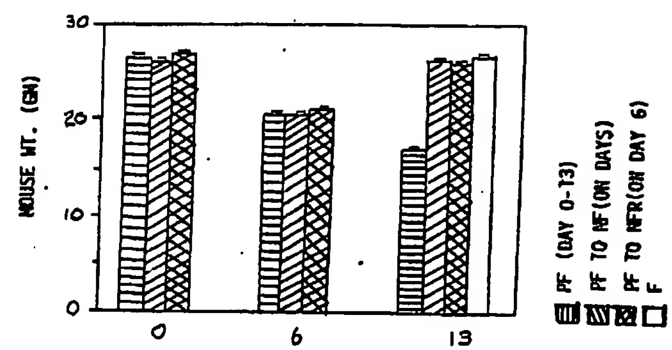
At least one drawing originally filed was informal and the print reproduced here is taken from a later filed formal copy.

- 1 -

2216416

FIG. 1

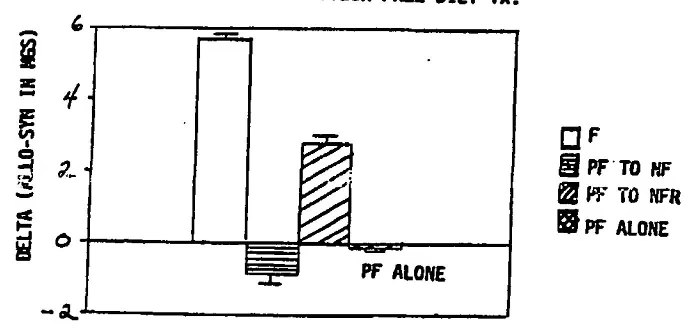
EFFECT OF PROTEIN FREE DIET ON MOUSE BODY WEIGHT



DAYS POST DIET MODIFICATION

FIG. 2

PLN DELTA VALUES AFTER PROTEIN FREE DIET TX.



DIET GROUPS

FIG. 3

DOSE RESPONSE PF⟶NFR, NFU--PLN

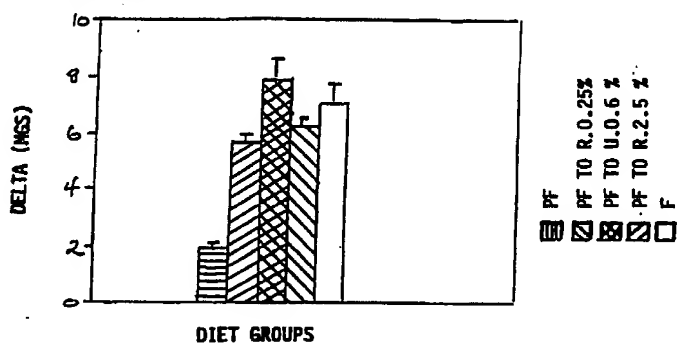
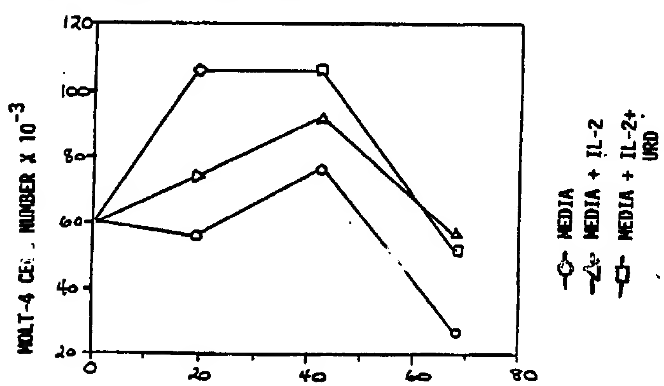


FIG. 4

MOLT-4 GROWTH ± NUCLEOSIDES



Case 610-7069

IMPROVEMENTS IN OR RELATING TO ORGANIC COMPOUNDS

The invention relates to the influence of nucleobases on the immune function.

Purines and pyrimidines are synthesised in cells with amino acids as the principal precursors. It has now been found that a depressed immune response in mammals can be reversed by the addition of a nucleobase source to the formulation.

The stimulating effect of nucleobase sources on the immune system has been demonstrated by tests, e.g. in mice having depressed T lymphocyte function due to protein-calorie malnutrition. Further animal tests show that the depressed immune function of animals placed on a protein free diet cannot sufficiently be restored by protein repletion - even though this allows restoration of body weight - but requires the administration of a nucleobase source.

The invention accordingly provides a method of stimulating the immune function in the human or animal body such as mammals (including humans), which comprises administering to a subject in need of such treatment an immune system stimulating amount of a nucleobase source. The nucleobase source may be administered by any conventional route in solid or liquid form, in particular enterally, e.g. orally or nasally, or parenterally, e.g. in the form of injectable solutions or suspensions.

For use according to the invention the nucleobase source may be administered in conventional pharmaceutcally or nutritionally acceptable formulation form.

Examples of conventional pharmaceutically acceptable formulation forms include tablets, capsules and injectable forms which may contain the active agent in admixture with conventional pharmaceutically

acceptable excipients, e.g. inert diluents or carriers, such as calcium carbonate, lactose and talc, granulating and disintegrating agents, e.g. starch and alginic acid, flavouring, colouring and sweetening agents, binding agents, e.g. starch or gelatin, lubricating agents, e.g. magnesium stearate and talc.

Examples of conventional nutritionally acceptable formulation forms include conventional diets, e.g. formula diets containing the nucleobase source in admixture with sources for essential amino acids and energy supply such as for proteins, carbohydrates and/or fat as desired.

Such dietary compositions may for example supply from 400 to 2000 Kcal, e.g. 1500 Kcal per day. The compositions of the inventions may be enriched with vitamins, such as vitamin C, minerals, such as iron, trace elements such as Selenium and optionally other elements, depending on the needs of the subject to be treated.

Formulations for enteral application are conveniently in solid or liquid form.

Nucleobase sources suitable for use in the method of the invention comprise, and more preferably consist esentially of natural nucleobases, nucleosides, nucleotides, RNA, DNA, equivalents thereof and/or mixtures comprising one or more of these compounds.

Natural nucleobases include the purines adenine and guanine as well as the pyrimidines cytosine, thymine and uracil.

Natural nucleosides include the ribose nucleosides adenosine, guanosine, uridine and cytidine and the deoxyribose nucleosides deoxyadenosine, deoxyguanosine, deoxythymidine and deoxycytidine.

Natural nucleotides include phosphate esters of natural nucleosides, such as the monophosphates adenylate (AMP), guanylate (GMP), uridylate

(UMP), cytidylate (CMP), deoxythymidylate (dTMP) deoxycytidylate (dCMP), and diphosphates and triphosphates of natural nucleosides such as ADP and ATP.

The amount of nucleobase source to be administered will i.a. depend on the type of treatment desired, e.g. whether prophylactic or therapeutic, and on the subject to be treated, e.g. the eating habits of the individual, whether the subject to be treated is a child or adult and the like. Thus, a heavy meat eater will have greater nucleobase source supply requirements than a person on a vegetable diet. In general, for larger mammals including humans, satisfactory results will be obtained with 1 to 50 times the normal daily amount of ca. 0.5 to 1.5g RNA corresponding with about 0.1 to 75g RNA, DNA, nucleosides or nucleotides per day or an equivalent amount in the form of nucleobases.

For the purpose of this invention one weight unit of nucleobase is regarded to be equivalent with 2.5 to 3 weight units of RNA, DNA, nucleosides or nucleotides. For convenience, the following daily amounts are expressed in g RNA only.

For long term or nutritional use, the daily amount of nucleobase source to be administered will conveniently vary within the range of from 0.1 to 4g RNA, preferably of from 1 to 3g RNA, in particular of from 1.5 to 2.5g RNA.

For short term or therapeutical use, the daily amount will in general be higher. For acute treatment with high amounts of nucleobase sources, it is preferred to employ pyrimidine nucleobase sources such as uridine or uracil. Preferred daily amounts for therapeutic use are from 100% to 2000% in excess of normal amounts, corresponding with from about 0.5g to 30g RNA. More preferred amounts for therapeutical use are in the range of from 1g to 20g RNA, in particular of from 1 g to 7.5g RNA per day. Pharmaceutical compositions may comprise a daily amount or parts thereof, e.g. in unit doses suitable for three or four

applications per day.

The method and compositions of the invention may be employed in any situation where a stimulation of the immune function is desirable, e.g. for restoring a normal immune response in a mammal with a deficient immune response, for abating the immunosuppressive effect of an immunosuppressant agent, for enhancing the development of the immune system in a developing mammal, for enhancing the activity of a senescent immune system of a mammal and the like.

In the following examples, tables and figures illustrating the invention

F is standard laboratory chow supplied by Purina under the code number 5008 and comprising 23.5% weight of a protein source from soybean, fish-bone meal and milk.

NF is a nucleotide free diet supplied by Purina under the code number 5755, comprising 21% by weight of casein as its protein source and only traces (less than 0.001% by weight according to HPLC analysis) of nucleotides. NF is isocaloric and isonitrogenous with F.

NFR is NF supplemented with RNA (purified yeast RNA).

NFR (0.25%) is NFR supplemented with 0.25% by weight purified yeast RNA.

NFA is NF supplemented with adenine.

NFU is NF supplemented with uracil.

PF is NF without protein, supplied by Purina under the code number 5765.

PLN stands for popliteal lymph nodes.

Fig. 1 represents the effect of protein free diet on mouse body weight;

Fig. 2 represents the PLN delta values after protein free diet tx.;

Fig. 3 represents the dose response PF — NFR, NFU — PLN; and

Fig. 4 represents the MOLT-4 growth ± nucleosides.

The stimulation index was calculated as follows:

$$\text{Stimulation index (S.I.)} = \frac{\text{Weight of allogeneically sensitized PLN in mg}}{\text{Weight of syngeneically sensitized PLN in mg}}$$

EXAMPLE 1

Dietary Nucleotides - Effect on Immunorestoration:

Mice were placed on a protein free diet (PF-Purina 5765). The animals were maintained on this diet for either six or eleven days and then switched to chow (F), 5755 (NF) or 5755 supplemented with various levels of RNA (NFR) or uracil (NFU).

Six to eight weeks after the swith of RF to F, NF, NFR or NFA all animals were use for the following in vivo assay:

The animals were inoculated with irradiated (3000R) 10^7 $C_{57}Bl/6$ allogenic spleen cells in the right hind footpad, while the left hind footpad received (3000R) 10^7 Balb/C syngeneic cells spleen as controls.

Seven days after the inoculations, mice were sacrificed and the popliteal lymph nodes (PLN) were excised and weighed. The weights of the mice in the 6 day experiment are shown in Fig. 1 and the PLN delta values plotted in Fig. 2. The actual data is give in Table 1. The F group was maintained on chow (F) throughout the experiment and was never given the protein free diet so they represent a normal control. This study shows that repletion of protein while restoring the body weight was not sufficient to restore any immune response in these mice while RNA addition gave considerable restoration of cellular immune function in a relatively short time period.

The same system was used to determine dose related effects of nucleotides. Mice were maintained on the protein free diet for 13 days and then converted to 5755 (NF) diet with 0.025% RNA (1/10 normal), 2.5% RNA (10X normal) or 0.6% uracil (10X normal) and the PLN assay started. The results are shown in Fig. 3 with data in Table 2, 4 and 5. In a separate experiment, shown in Table 3, a group of mice on the

protein free diet were switched to chow diet and compared to 2.5% RNA and 0.6% uracil groups. The recovery of activity was significantly less in chow than in both high level nucleotide groups. It is clear from these studies that higher levels of uracil or RNA than normally found in the diet were more effective in restoring lost immune function.

TABLE 1

Protein Free Diet Induced Immunosuppression And Its Reversibility With Dietary Nucleotides.

Diet	Allogenic lymphnode(mgs)	Syngenic lymphnode(mgs)	Delta[1]	Stim. Index[2]
F	10.7	3.1	7.6	3.45
	9.3	4.0	5.3	2.33
	8.4	2.0	6.4	4.20
	6.2	2.3	3.9	2.69
	6.5	1.4	5.1	4.64
X±SEM	8.2±0.9	2.6±0.5	5.7±0.6	3.5±0.4
PF to NF	1.6	1.9	-0.3	0.84
	1.7	1.7	0.0	1.00
	1.6	2.0	-0.4	0.80
	1.2	2.2	-1.0	0.55
	2.5	1.2	1.3	2.08
X±SEM	1.7±0.2	1.8±0.2	-0.8±0.4	1.1±0.3
PF to NFR	4.3	0.8	3.5	5.38
	3.7	1.8	1.9	2.05
	3.1	0.6	2.5	5.16
	4.6	1.2	3.4	3.83
	2.8	0.4	2.4	7.00
X±SEM	3.7±0.3	1.0±0.3	2.8±0.3	4.7±0.8
PF alone	0.66	0.90	-0.24	0.73
	0.72	0.92	-0.20	0.78
	0.94	0.75	0.19	1.25
	0.85	1.00	-0.15	0.85
	1.45	1.10	0.35	1.32
X±SEM	0.9±0.1	0.9±0.06	-0.01±0.1[a]	1.0±0.1

1: Delta=allo lymphnode minus syn lymphnode
2: Stim.Index=S.I.= allo lymphnode divided by syn lymphnode weight
a: PF VS F, PF to NFR p<0.002
VS PF, NF p<0.89

TABLE 2

Body weights(gms) of Day 18 PLN experiment

Diet group	Day 1	Day 11	Day 18
PF	23.5±0.7	18.2±1.3	16.6+0.9
PF-NFR(0.025%)	22.8±1.7	18.3±1.0	23.1±1.5
PF-NFR(2.5%)	23.0±2.5	18.5±2.1	23.5±2.7
PF-NFU(0.6%)	22.6±1.9	17.3±1.3	24.4±1.9
F	22.0±0.8	22.7±0.9	23.0±0.9

Day 1 - mice started on PF diet.
Day 11 - diet switch and PLN assay set up.
Day 18 - PLN harvest and results.

TABLE 3

Dietary Nucleotide Dose Response Following PF Induced Immunosuppression.

Diet group	Allo LNs (mgs)	Syn LNs (mgs)	Delta (allo-syn)	Stim.Index (allo/syn)
PF	3.3	0.7	2.6	4.7
	4.2	1.0	3.2	4.2
	2.9	2.0	0.9	1.4
	4.0	1.3	2.7	3.1
	0.9	0.9	0.0	1.0
	3.1±0.6	1.2±0.2	1.2±0.6	2.9±0.7
PF--NFR(0.025%)	6.5	1.3	5.2	5.0
	8.6	1.8	6.8	4.8
	8.7	2.0	6.7	4.3
	7.7	2.1	5.6	3.7
	5.6	1.5	4.1	3.7
	7.4±0.6	1.7±0.2	5.7±0.5	4.3±0.3
PF--NFR(2.5%)	9.7	1.5	8.2	6.5
	5.9	1.3	4.6	4.5
	4.7	1.9	2.8	2.5
	7.2	1.0	6.2	7.2
	7.4	1.2	6.2	6.2
	7.0±0.8	1.4±0.2	5.6±0.9	5.4±0.8
PF--NFU(0.6%)	10.3	1.5	8.8	6.7
	9.2	1.5	6.7	6.1
	9.5	1.6	7.9	5.9
	13.0	2.1	11.1	6.2
	6.8	1.6	5.2	4.2
	9.8±1.0	1.7±0.3	7.9±1.0	5.8±1.0
F--F	6.0	1.8	4.2	3.3
	11.6	1.4	10.2	8.3
	8.3	2.2	6.1	3.8
	6.2	0.7	5.5	8.9
	10.9	1.2	9.7	3.1
	8.6±1.2	1.5±0.3	7.1±1.2	6.7±1.3

AlloLNs: C_{57}Bl/6 lymphnodes, SynLNs: Balb/c lymphnodes.
PF started day one, dosaged diets and PLN assay day 11
LNs harvested day 18

	Delta	Stim.Index
PF vs PF--NFR0.025%	p<0.01	N.S.
vs PF--NFR2.5%	<0.01	<0.05
vs PF--NFU0.6%	<0.001	<0.05
vs F	<0.01	<0.05

TABLE 4

Dietary Nucelotide Dose Response Following
PF Induced Immunosuppression.

Diet group	Allo LNs (mgs)	Syn LNs (mgs)	Delta (allo-syn)	Stim.Index (allo/syn)	p
PF	1.8	0.5	1.3	3.6	
	1.6	0.8	0.8	2.0	
	1.0	0.6	0.4	1.7	
	1.6	1.9	-0.3	0.8	
	2.2	1.2	1.0	1.8	
Avg ± SEM	1.6 ± 0.2	1.0 ± 0.3	0.6 ± 0.3	2.0 ± 0.5	>0.1
PF--F	3.1	1.5	1.6	2.1	
	3.6	1.4	2.2	2.6	
	5.4	2.0	3.4	2.7	
	4.7	1.1	3.6	4.3	
	10.4	3.6	6.8	2.9	
	5.4 ± 1.3	1.9 ± 0.4	3.5 ± 0.9	2.9 ± 0.4	>0.2
PF--NF	2.3	1.6	0.7	1.4	
	4.1	2.0	2.1	2.1	
	6.1	2.5	3.6	2.4	
	3.7	2.8	0.9	1.3	
	3.6	1.4	2.2	2.6	
	4.0 ± 0.6	2.1 ± 0.3	1.9 ± 0.5	2.0 ± 0.6	
PF--NFR (0.025%)	4.2	1.5	2.7	2.8	
	3.8	2.5	1.3	1.5	
	3.5	1.5	2.0	2.3	
	3.4	1.4	2.0	2.4	
	4.2	1.8	2.4	2.3	
	3.8 ± 0.8	1.7 ± 0.2	2.1 ± 0.2	2.3 ± 0.2	>0.8
PF--NFR (0.25%)	5.1	2.8	2.3	1.8	
	3.9	1.5	2.4	2.6	
	3.9	1.7	2.2	2.3	
	5.6	1.8	3.8	3.1	
	6.4	1.3	5.1	4.9	
	5.0 ± 0.5	2.0 ± 0.3	3.2 ± 0.6	2.9 ± 0.5	>0.2
PF--NFR (2.5%)	7.0	2.7	4.3	2.6	
	8.7	3.6	5.1	2.4	
	8.5	3.1	5.4	2.7	
	6.2	3.0	3.2	2.1	
	7.4	2.8	4.6	2.6	
	7.6 ± 0.5	3.0 ± 0.2	4.5 ± 0.4	2.8 ± 0.1	>0.01
PF--NFU (0.06%)	4.6	2.4	2.2	1.9	
	4.4	1.6	2.8	2.8	
	5.5	0.9	4.6	6.1	
	9.2	2.1	7.1	4.4	
	8.2	2.6	5.6	3.2	
	6.4 ± 1.0	1.9 ± 0.3	4.9 ± 0.9	3.7 ± 0.7	>0.01

TABLE 4 (con't)

PF--NFU (0.6%)	4.2	0.5	3.7	5.4	
	4.9	1.3	3.6	3.8	
	5.5	1.4	4.1	3.9	
	6.2	1.5	4.7	4.1	
	6.3	1.6	4.7	3.9	
	5.4 ± 0.4	1.3 ± 0.2	4.2 ± 0.2	4.8 ± 0.9	>0.01
F--F	8.3	1.7	6.6	4.9	
	8.8	2.2	6.6	4.0	
	8.7	2.7	6.0	3.2	
	6.8	1.9	4.9	3.6	
	6.4	2.1	4.3	3.0	
	7.8 ± 0.5	2.1 ± 0.2	5.7 ± 0.5	3.7 ± 0.8	>0.001

AlloLNs: C_{57}Bl/6 lymphnodes
SynLNs: Balb/c lymphnodes
PF started on day 1, dosaged diets and PLN assay day 8, LNs harvested on day 16
p values = PF--NF vs individual groups (calculated by student T test)

TABLE 5

Body Weights (gms) of Day 16 PLN Experiment

Diet group	Day 8	Day 12	Day 16
PF	15.2 ± 0.9	17.2 ± 0.6	13.7 ± 0.6
NFU (0.6%)	19.7 ± 0.7	22.0 ± 0.9	21.7 ± 0.7
NFU (0.06%)	18.8 ± 0.5	22.3 ± 0.6	19.4 ± 1.1
NFR (2.5%)	18.6 ± 0.9	21.7 ± 0.4	22.5 ± 0.5
NFR (0.25%)	18.7 ± 0.3	21.6 ± 0.5	20.2 ± 0.6
NFR (0.025%)	17.2 ± 0.6	20.5 ± 0.6	19.8 ± 0.6
NF	19.7 ± 0.8	23.3 ± 0.9	22.5 ± 0.8
PF--F	18.5 ± 0.3	23.2 ± 0.4	22.1 ± 0.4
F	23.6 ± 0.7	23.3 ± 0.4	21.5 ± 0.6

EXAMPLE 2

MOLT-4 Human T-Lymphoblast Growth in Serum Free RPMI-1640 + Human IL-2 + Nucleosides In Vitro:

Experiments have shown that the murine IL-2 dependent helper T-cell line (HT-2) requires nucleosides (uridine and inosine) for optimal G_1 to S phase transition in serum free media. Uridine alone was particularly effective. To determine nucleoside requirement in human cultured T-cell lines, preliminary growth kinetic experiments using the human T-lymphoblast cell line MOLT-4 were run. This cell line produces IL-2 after stimulation with lectins and phorbol esters. Long-term culture of this cell line is not Il-2 dependent but responds to IL-2. MOLT-4 cells were arrested at G_0/G_1 phase following extensive washing and removal of Fetal Bovine Serum. Cell cultures were planted at a cell density of $5X10^4$ cells/ml RPMI-1640 with 20 µM hepes and 2 mM L-glutamine. Human IL-2 (5 µ/ml) and uridine (100 µM) was added to cell cultures at time 0 and viable cell number was monitored at various time points for 72 hours.

The results (Fig. 4) show that adding uridine (100 µM) to serum free MOLT-4 cultures in the presence of IL-2 increased G_1-S phase transition in terms of viable cell number and over a time course (i.e., G_1-S phase transition occurred at 20 hours in cultures with uridine and 40 hours in cultures without uridine). Other experiments show that adenosine + inosine + uridine in combination is more effective than uridine alone.

CLAIMS

1. The use of a nucleobase source for the stimulation of the immune function in the human or animal body.
2. The use according to Claim 1 in the form of a dietary composition for humans.
3. The use according to Claim 1 in the form of a pharmaceutical composition for humans.
4. The use of a nucleobase source for the manufacture of a medicament or of a dietary composition for the stimulation of the immune function in the human body.
5. A method of stimulating the immune function in the human body, which comprises administering to a subject in need of such treatment an immunostimulatory amount of a nucleobase source.
6. The method of Claim 5 for restoring a normal immune response in a human with a deficient immune response, or for abating the immunosuppressive effect of an immunosuppressive agent, or for enhancing the development of the immune system in a developing human or for enhancing the activity of a senescent immune system.
7. The use or method of any one of Claims 1 to 6 comprising administering from 0.1 to 75 g RNA, DNA, nucleotides or nucleosides per day or an amount equivalent thereto in nucleobase form.
8. The use or method of Claim 7, wherein the nucleobase source is administered in the form of a dietary composition comprising from 0.1 to 4 g, more preferably 1 to 3 g, in particular 1.5 to 2.5 g of RNA, DNA, nucleosides or nucleotides per administration unit for a day, or nucleobases in an amount equivalent thereto.
9. The use or method of Claim 7, wherein the nucleobase source is administered in the form of a pharmaceutical composition comprising from 0.5 g to 30 g preferably from 1 to 20, in particular from [illegible] to 7.5 g RNA, DNA, nucleotides or nucleosides per administration unit for a day, or nucleobases in an amount equivalent thereto, whereby said administration unit may be administered as such once a day or in the form of sub-units several times a day.

10. The use or method of Claim 9 for treatment of states of immuno-suppression requiring treatment with elevated daily amounts of a nucleobase source, comprising the administration of a pyrimidine nucleobase source, such as uridine or uracil.
11. A pharmaceutical or dietary composition comprising an immuno-stimulatory amount of a nucleobase source.
12. The pharmaceutical composition of Claim 11, comprising conventional, pharmaceutically acceptable excipients in enteral or parenteral application form.
13. The dietary composition of Claim 12, comprising sources for essential amino acids and energy supply in enteral or parenteral application form.
14. The composition of Claims 11 to 13, comprising one or more of the components selected from vitamins, minerals and trace elements.
15. The composition of Claims 11 to 14, comprising from 0.1 to 75 g of RNA, DNA, nucleotides or nucleosides per administration unit per day or an amount equivalent thereto in nucleobase form, whereby such administration unit may be divided over shaped sub-units, e.g. tablets or injectables, where more than one administration per day is indicated.
16. A dietary composition of Claim 15, comprising from 0.1 to 4.0 mg, preferably 1 to 3 g, RNA, DNA, nucleosides or nucleotides per administration unit for a day, or an amount equivalent thereto in nucleobase form.
17. A pharmaceutical composition of Claim 15, comprising from 0.5 to 30 g, preferably from 1 to 20, in particular from 1 to 7.5 g RNA, DNA, nucleotides or nucleosides per administration unit for a day, or an amount equivalent thereto in nucleobase form.
18. A pharmaceutical composition of Claim 17, wherein the nucleobase source is a pyrimidine nucleobase source such as uridine or uracil.
19. The use, method or composition of any of Claims 1 to 5 and 15 to 18, wherein the employed nucleobase source is selected from natural nucleobases, natural nucleosides, natural nucleotides, RNA, DNA, equivalents of these components and/or mixtures

comprising two or more of such components.
20. Process of preparing a composition of Claims 11 to 18 comprising admixing the nucleobase source with conventional, pharmaceutically acceptable excipients and/or with sources for essential amino acids and for energy supply, and with optionally vitamins, minerals, trace elements and converting the mixture in the desired enteral or parenteral application form.

Published 1989 at The Patent Office, State House, 66/71 High Holborn, London WC1R 4TP. Further copies may be obtained from The Patent Office. Sales Branch, St Mary Cray, Orpington, Kent BR5 3RD. Printed by Multiplex techniques ltd, St Mary Cray, Kent, Con. 1/87